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US ARMY MEDICAL RESEARCH LABORATORY

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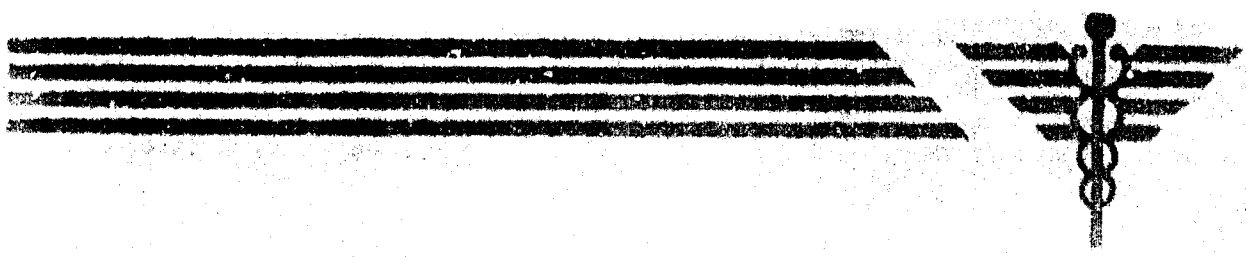
REPORT NO. 649

PRECIPITIN AND NEUTRALIZING ANTIBODY RESPONSE ELICITED BY CROTALUS ATROX VENOM ANTIVENOM PRECIPITATE

by

A. J. Luzzio, Ph. D.
and
Major G. S. Treviño, VC

30 November 1965



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ABSTRACT

PRECIPITIN AND NEUTRALIZING ANTIBODY RESPONSE ELICITED BY CROTALUS ATROX VENOM ANTIVENOM PRECIPITATE

OBJECT

To determine the relationship between C. atrox neutralizing and precipitin antibody and the ability of venom-antivenom complexes to stimulate venom neutralizing antibody.

RESULTS AND CONCLUSIONS

Rabbits immunized with insoluble C. atrox venom-antivenom precipitate produced less precipitins and nearly as much neutralizing antibody as those injected with whole venom. The data show that venom neutralizing antibodies are precipitins and that the apparent non-relationship between precipitin titer and antitoxic value is due to the polyvalency of venom-antivenom systems. The value of using insoluble antigen-antibody precipitates to isolate specific antigen, from a mixture, for immunization is pointed out.

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INTRODUCTION

Diphtheria toxin neutralized with antitoxin was used as an immunizing agent in 1898 (1). Other reports followed which indicated that such antigen-antibody complexes were unique in that antibody formation to the toxin was enhanced when compared to using the toxin alone (2-4). Similar results were reported for tetanus and serum albumin complexed with specific antiserum (4-8).

This ability of insoluble antigen-antibody complexes, in enhancing antibody formation, could have a twofold advantage with extremely toxic antigens. First from the added protection gained by increased antibody, and secondly, because of slower release, it is likely that more antigen could be injected, bound to antibody, than could be given alone without deleterious effects. For example, the greatest difficulty experienced with producing rapid and effective immunity to snake venoms is inherent in the extreme toxicity of the venom and its high resistance to chemical and physical detoxifying agents. These factors dictate the use of venoms in extremely low doses over a prolonged period of time to allow for the introduction of sufficient antigen to establish effective immunity.

One of the requirements for the formation of an insoluble antigen-antibody complex is that the system be precipitable. Bier (9) showed that the quantitative course of the precipitin reaction is very similar for Crotalus terrificus venom-antivenom and diphtheria toxin-antitoxin systems. However, subsequent to an earlier report that rabbits immunized with cobra venom produce precipitating antibodies (10), it was reported that no constant relationship existed between antitoxic value and precipitating power of rabbit anti-cobra venom (11).

In the present investigation rabbits were inoculated with Crotalus atrox venom-antivenom precipitates, and the sera were analyzed for precipitating and neutralizing antibodies. Evidence is presented which shows that neutralizing, as well as precipitating, antibody was stimulated by the precipitate complex, that venom neutralizing antibody is a precipitin, and that the apparent non-relationship between precipitin content and antitoxic value is due to the polyvalency of the venom-antivenom systems. The data support the use of antigen-antibody

complexes as a method for isolating specific antigens from a mixture for immunizing purposes.

MATERIALS AND METHODS

Antigen Preparations and Immunization Procedure. C. atrox venom was extracted from a large number of snakes, maintained at the US Army Medical Research Laboratory, by allowing them to inject the venom directly through a latex diaphragm into an ice cooled container which was then frozen at -15°C. The pooled venom was then lyophilized and maintained in the dried state in a desiccator at -15°C. For rabbit inoculations the dried venom was brought directly into solution with 4 per cent sodium alginate adjuvant (Colab Laboratories, Chicago, Illinois). Unused aliquots were stored at -15°C and thawed, when needed, for additional inoculations. Venom antigen for precipitin and neutralizing antibody titrations was made up fresh, from the dried state, with saline.

Initial venom-antivenom complex was prepared by precipitating 1 ml commercial equine origin antivenom (Crotalidae polyvalent, Wyeth Laboratories, Inc., Marietta, Pennsylvania) with .3 mg C. atrox whole venom N (venom nitrogen) after preliminary titrations indicated this ratio gave maximum precipitation. The precipitate was washed three times with saline and then resuspended in 4 per cent sodium alginate adjuvant.

Five New Zealand white rabbits, obtained locally, were each injected subcutaneously with three doses of 10 mg TP (total protein) initial venom-antivenom complex given one day apart (total 30 mg). The animals were rested for seven days and the same series of injections were repeated. After a second rest period of one week, each rabbit was inoculated with a third series consisting of a total of 9 mg (dry wt.) whole C. atrox venom given in three equal doses one day apart.

Blood was collected from each rabbit by cardiac puncture eight days after the last inoculation, was allowed to clot at room temperature, and the serum was then separated by centrifugation. A preliminary quantitative precipitin titration determined that a ratio of 1 ml of this antiserum to 50 μg whole C. atrox venom N (slight antigen excess) produced maximum precipitation. Rabbit antivenom-venom complexes were then precipitated using this ratio, washed three times with saline and finally resuspended in Na alginate for the following experimental

animal inoculations. Aliquots not used immediately were stored in the same manner as whole venom in adjuvant.

Immunization: Primary Response. Each animal in a group of 10 rabbits (Group I) was inoculated subcutaneously with a total of 0.48 mg whole C. atrox venom N. Three equal doses were given one day apart. At the same time, each of 10 rabbits (Group II) was inoculated with a total of 2.28 mg complex N also given in three equal doses. Based on the quantitative precipitin titration, the calculated maximum value for bound venom in the inoculated complex was equivalent to 0.36 mg whole venom N. Rabbits were bled from the marginal ear vein at periodic intervals after the last inoculation and the pooled serum, from two samples of five rabbits each, was titrated as a single specimen for precipitating and neutralizing antibody. These results were averaged in the final data processing.

Immunization: Secondary Response. Group I and Group II rabbits were started on a second series of inoculations 34 days after the last dose of the primary series via the same route and schedule as the first series. Group I rabbits were given a total of 0.96 mg whole C. atrox venom N while each of the rabbits in Group II was given a total of 5.0 mg complex N equivalent to a theoretical maximum of 0.78 mg of bound whole C. atrox venom N. The animals were periodically bled from the marginal ear vein after the last inoculation and the serum was again analyzed for precipitating and neutralizing antibody. Three rabbits (Group III) were maintained and bled along with the experimental group throughout the first and second series as uninoculated controls. The pooled serum from these rabbits was also treated as a single sample.

Quantitative Precipitin Titrations. Complete details of the quantitative precipitin technique may be found in Kabat and Mayer (12). Antigen-antibody precipitates and antigen solutions were analyzed for protein N by the Lowry modification of the Folin-Ciocalteau procedure (13).

Neutralizing Antibody Determinations. Whole C. atrox venom in physiological saline was made up in a stock concentration of 2 mg per ml (dry wt.), and .2, .4, .6 and .8 ml were added to 1.0 ml of pooled test serum from Groups I, II and III, and the volume of each dilution was brought to 4.0 ml with physiological saline. After 1 hour incubation at 37°C, 0.5 ml of each dilution was injected intraperitoneally into Swiss albino mice weighing between 18 and 20 gms. In these titrations the pooled sera from all animals in their respective groups

were treated as a single specimen. Five test animals were used for each venom-serum dilution containing 50, 100, 150 and 200 micrograms of venom. The number of live and dead mice was recorded during a 24 hour period and the LD_{50} was then calculated according to the method of Reed and Meunch (14).

RESULTS

Primary Response. Neutralizing antibody was measurable only on the second day following the last inoculation. Preliminary determinations showed that the quantity of whole venom in saline required for a one LD_{50} dose in mice was 75 µgms (dry wt.). In the presence of .125 ml of normal rabbit serum (Group III) 86 µgms of the same toxin were required to produce a one LD_{50} dose in mice, suggesting slight neutralization of venom by normal rabbit serum. In the presence of .125 ml of sera from Groups I and II, 116 and 150 µgms of toxin, respectively, were necessary for a one LD_{50} dose in mice. Thus, by subtraction, it is apparent that antiserum from Group I neutralized 41 µgms of toxin and that from Group II neutralized 75 µgms.

The same sera when tested by quantitative precipitation were found to be non-reactive with whole venom.

Secondary Response. The data in Figure 1 show that rabbits inoculated with whole venom (Group I) reached peak precipitin titer five days after the last dose of the second series inoculations. Thereafter, there was a precipitous fall in antibody. The neutralizing antibody reached peak titer at three days and did not decline as rapidly. At this time 180 µgms of whole venom in the presence of .125 mg of Group I serum were required to produce a one LD_{50} dose in mice as compared to 86 µgms in the presence of .125 ml of serum from uninoculated controls (Group III). Rabbits inoculated with venom-antivenom complex (Group II) produced considerably less precipitins, but neutralizing antibody reached peak titer at five days. At this time 175 µgms of whole venom in the presence of .125 ml of Group II serum were required for a one LD_{50} dose in mice.

Even though Group II serum was only slightly less than Group I serum in neutralizing antibody, the more rapid appearance and longer persistence of antibody in the latter group was typical for a secondary immune response, whereas the data for Group I were more in line with that expected from a primary response. This result was indicative of less antigenic stimulation in the Group II rabbits.

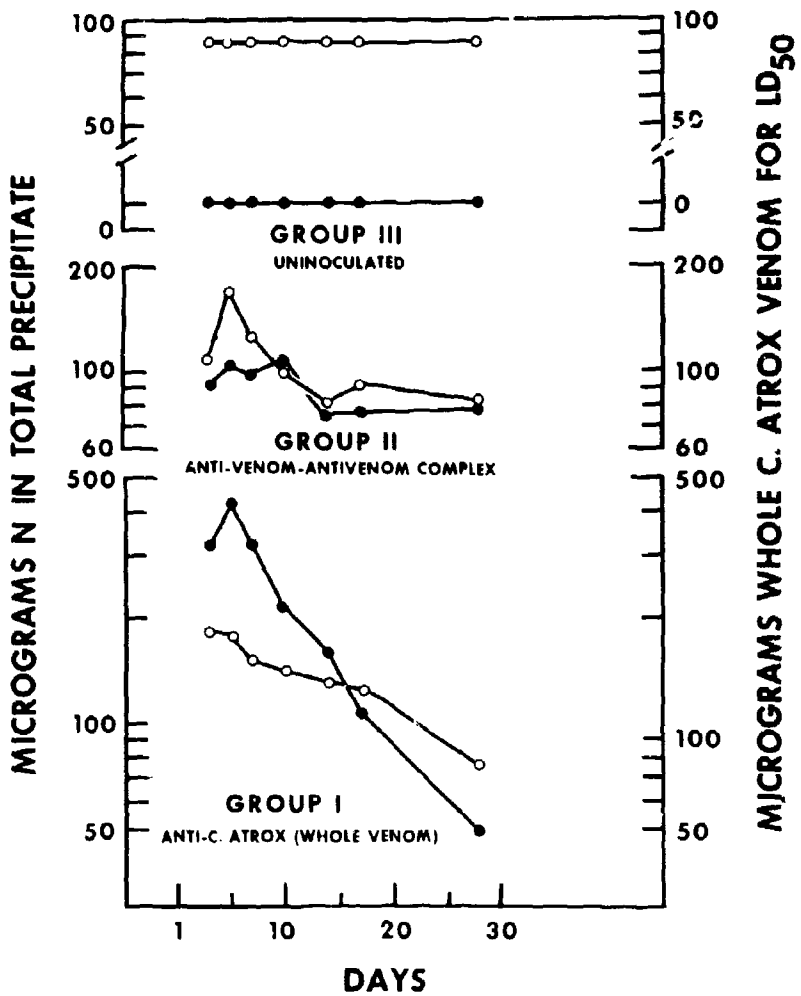

Fig. 1. Average precipitin response (● solid circles) and neutralizing antibody (o open circles) in 10 rabbits inoculated with whole C. atrox venom (Group I), 10 rabbits inoculated with rabbit venom-antivenom complex (Group II) and 3 uninoculated controls (Group III).

DISCUSSION

A twofold increase in serum neutralizing antibody was elicited by injecting rabbits with whole C. atrox venom, and a slightly lesser increase was obtained with venom-antivenom precipitates. It is

obvious that this yield would have been considerably amplified, possibly as much as tenfold, had the venom been titrated against the concentrated purified globulins as is often done with antivenoms. Inoculations with whole venom resulted in considerably more precipitins than were elicited by the complex. From calculations based on the amount of crude venom added to rabbit antivenom and the total N precipitated a maximum of 1.14 mg bound venom N was given each rabbit of Group II in six doses, while 1.44 mg whole venom N was injected into each of the animals in Group I. However, at slight antigen excess not all the whole venom antigens added to the antivenom appeared in the final precipitate complex. Thus, the actual amount of bound venom injected into each rabbit of Group II was much lower than the calculated value of 1.14 mg and qualitatively different than whole venom. Glenn et al. (15) reported that at least 16 precipitin bands occurred when whole venom-polyvalent horse anti-crotalidae systems were analyzed by double diffusion. With monovalent antivenoms Minton (16) showed that rattlesnake venoms are composed of at least 4-7 distinct antigenic fractions. Thus, it is evident that venom-antivenom titrations constitute multivalent systems in which, at all dilutions, some systems are either at antigen or antibody excess. The result is that at equivalence, or at slight antigen excess, only a portion of the antigen mixture is precipitated with antivenom.

From the data given above and the results shown in Figure 1 it is apparent that the insoluble venom-antivenom precipitate, even though rich in lethal toxin, contained fewer antigens, than whole venom. Since antibody response is, in part, dose dependent it is likely that the higher precipitin titer of Group I animals resulted from the higher protein concentration, and the greater number of different antigens in whole venom as compared to the complexed venom. The nearly equivalent response of Group I and Group II rabbits in neutralizing antibodies may be explained by the greater specificity for lethal toxin of the injected complex. This is ample evidence that venom neutralizing antibodies are precipitins. Furthermore, the neutralizing antibody of Group II more closely follows the precipitin pattern in that both reached peak titer at the same time. The observed increase in precipitins on the tenth day is not consistent with the progressive decline that usually occurs with pure antigen-antibody systems. This may occur because of the peculiarities inherent in using complexed antigen, e.g., the slow release of bound antigen by dissociation from the complex.

SUMMARY

Rabbits immunized with insoluble C. atrox venom-antivenom precipitate produced less precipitins and nearly as much neutralizing antibody as those injected with whole venom. The data show that venom neutralizing antibodies are precipitins and that the apparent non-relationship between precipitin titer and antitoxic value is the result of the polyvalency of venom-antivenom systems. The value of using insoluble antigen-antibody precipitates to isolate specific antigens, from a mixture, for immunization is pointed out.

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